USDA ARS National Animal Germplasm Program

Ram Semen Processing, Cryopreservation, Estrous Synchronization, and Non-surgical Artificial Insemination (AI) Protocol

Please read all instruction and recommendations prior to attempting any of the techniques.

Recommendations for successful non-surgical artificial insemination:

The breed of the ewe is a source for variation for the fertility resulting from these techniques. It is recommended that a small number of ewes are initially inseminated, and at various times, to determine an optimal time to AI for that flock and breed. We suggest initially attempting single inseminations at 47, 49, 51 and 53 hours post-CIDR removal to determine if there is an optimal time to AI. We also recommend that expensive, imported ram semen is not used until an optimal AI time with these techniques is be determined for the flock and breed.

Rams used for semen collections should be at least 2 years old. If using an artificial vagina for semen collections, rams that have live-covered ewes will be more willing to be collected via artificial vagina. This will also make the collections more productive (more samples of a higher quality) and minimize stress on the animals.

Ewes should be aged 4 to 6 years. If additional animals are needed, then the next age that should be included are 2 to 3-year-old ewes. Preferably, the ewes will have at least 1 parity.

Semen collection and processing:

Collect semen from sexually mature rams via an artificial vagina (preferred) or electroejaculation.

Inspect sample to ensure it is free of urine and other contaminants.

Determine the sperm concentration and motility using spectrophotometry and a Hamilton Thorne motility analyzer (Beverly, MA), respectively (at least 5 fields of analysis and 500 sperm) or microscopy and a hemocytometer.

Freeze semen samples with either skim milk egg yolk (SMEY) cryopreservation medium or Tris-egg yolk-glycerol (TEYG) cryopreservation medium. Both media enable acceptable fertility to be achieved when non-surgical artificial insemination (AI) is performed. However, the TEYG medium (known commercially as Biladyl or Triladyl from Minitube, USA) is easier to prepare and samples can be held in this medium for at least 24 hours prior to freezing with only minimal effects on the sperm, post thaw motility, and fertility. **Warning:** *SMEY is not a suitable medium for holding sperm for long periods (greater than 3 hours) prior to cryopreservation.*

If SMEY cryopreservation medium is used, then:

Dilute semen samples to 1200 x 10^6 sperm/mL in 37 °C skim milk-egg yolk ***cooling*** media (see recipe below).

Place the samples in a 37 °C water jacket and cool to 5 °C over 45 to 60 min.

Dilute the samples drop-wise over 5 min (1:1; volume to volume) with 5 °C skim milk-egg yolk ***freezing*** media (see recipe below) resulting in a final sperm concentration of 600 x 10^6 sperm/mL.

Load into 0.5 mL semen straws and freeze.

If the TEYG freezing medium is used, then:

Dilute the samples slowly, in one step, with the TEYG freezing medium to 400 x 10^6 sperm/mL.

Cool the sample to 5 °C over 90-120 minutes.

If necessary, once the samples are cooled to 5 °C they can be maintained at this temperature for up to 48 hours prior to freezing to enable transportation via overnight courier to a centralized laboratory. However, it is always prudent to cryopreserve samples as soon as possible following collection to maximize post-thaw quality and fertility.

Load the samples into 0.5 mL semen straws and freeze.

Semen cryopreservation and thawing:

Samples are frozen by:

Box freezing: Samples are placed on a rack and frozen in liquid nitrogen vapor (4 cm above liquid nitrogen) for 10 min.

Plunge into liquid nitrogen for storage.

-OR-

Programmable freezer: Freeze samples using a programmable freezer (e.g. the Cryo Bio System Mini Digitcool UJ400, IMV Corporation, Minneapolis, MN) and the following curve: 5 °C to -10 °C at 5 °C/min; -10 °C to -130°C at 60 °C/min.

Plunge into liquid nitrogen for storage.

Samples cryopreserved using either the SMEY or TEYG are thawed for 30 seconds in a 37 °C water bath. Ensure that the straws are completely dry before proceeding with a motility analysis or AI because water will kill the sperm sample.

Estrous synchronization:

Synchronize the estrous cycles of ewes using:

Sponges for 14 days (e.g. Chronogest CR containing 40 mg fluorogestone acetate, Intervet, Milton Keynes, UK) followed by PMSG (400 IU, i.m.; total volume = 4mL from an 18 gauge needle; single injection) administered 48 hours prior to sponge removal;

-OR-

CIDRs (e.g. 0.3 g progesterone in an inert silicone elastomer for 12 days; Pfizer Animal Health, New York, NY) followed by PMSG (400 IU, i.m.; total volume = 4mL from an 18 gauge needle; single injection) administered 24 hours prior to, or at the time of sponge removal;

Non-surgical artificial Insemination:

Insemination dose and timing

The standard sperm dose is 100 x 10^6 motile sperm inseminated at 53- and again at 57-hours post CIDR removal.

If identification of an optimal AI time using a single insemination is being performed, then a minimum of 70 x 10^6 motile sperm in a single dose should be used. This will not result in maximal fertility but will help to identify a range of times within which to inseminate. Then, once the optimal insemination time is identified, a double insemination 4 hours apart can be used to maximize fertility.

Artificial insemination

Restrain ewes in a standing position in a sheep handling squeeze chute in order to minimize stress. As an alternative, a haltered ewe can be held at the shoulder and hip next to a panel or wall.

Apply non-spermicidal lubricant, about 1 cm^3, to the tip of the AI gun (e.g. All-2-Mate Goat Gun, Continental Plastics, Delavan, WI) and place the lubricant on the bottom of the interior of the labia.

Part the labium and insert the AI gun upward at a 45° angle through the lubricant.

Once contact is made with the top, interior of the vagina, tilt the AI gun into a horizontal position and continue to gently insert the gun further into the vagina without force.

Once resistance to insertion of the AI gun is observed, pull the gun back about 3 cm and probe with the tip in different directions to determine if the gun can be inserted further. On some occasions the gun will enter the cervix and the technician can feel the resistance and eventual passing of the cervical rings. Additional probing is attempted to determine if a deeper insemination may be achieved.

When the technician determines that the maximum depth with the AI gun is achieved the insemination dose is slowly deposited and the gun is removed from the ewe. Remember: *the goal is to deposit the insemination dose as deep as possible in the vagina/cervix of the ewe without cervical manipulation or force.*

Recipes

SMEY Cryopreservation Medium Recipe from Paulenz et al., 2007

Skim milk-egg yolk ***cooling*** medium

Dilute 11 grams of non-fat dried skim milk into approximately 80 mL of distilled/deionized water and heat this solution to 95 °C for 10 min. Allow the solution to cool to room temperature and then add 5 mL of egg yolk, 1mg/ml streptomycin sulfate and sufficient water to bring the final volume to 100 mL. Mix the solution until homogeneous.

Skim milk-egg yolk ***freezing*** medium

86% SMEY Cooling Medium by volume
14 % glycerol by volume.

TEYG Cryopreservation Medium Recipe from Davis et al., 1963:

This is a 1-step dilution medium and is also commercial available under the names Biladyl or Triladyl. This recipe makes 500 mL of ram semen cryopreservation diluent.

TRIS (MW 121)	12.112 g
Citric acid	6.8g
Glucose	5.0g
Glycerol	25.0 mL

Fill to 400 mL with distilled, deionized water
Egg yolk 100 mL (20% by volume)
Antibiotics (either CSS specifications or at least 500 mg streptomycin sulfate)

Ram semen motility evaluation medium:

200 mM Tris
65 mM citric acid monohydrate
55 mM glucose

References:

Davis, I.S., Bratton, R.W., Foote, R.H., 1963. Livability of bovine spermatozoa at 5 C in Tris-buffered and citrate-buffered yolk-glycerol extenders. J. Dairy Sci. 46, 57-60.

Heiko Paulenz, Tormod Ådnøy and Lennart Söderquist . 2007. Comparison of fertility results after vaginal insemination using different thawing procedures and packages for frozen ram semen. Acta Veterinaria Scandinavica. 49:26.

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